AD_____

Award Number: DAMD17-98-1-8209

TITLE: Role of Cyclin D1 and cdk Inhibitors in Breast Cancer Pathogenesis

PRINCIPAL INVESTIGATOR: Kristin Roovers, Ph.D.
R. K. Assoian, Ph.D.

CONTRACTING ORGANIZATION: The University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

REPORT DATE: May 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2002	Annual Summary (30 Sep 98 - 30 Apr 02)

4. TITLE AND SUBTITLE
Role of Cyclin D1 and cdk Inhibitors in Breast Cancer Pathogenesis

5. FUNDING NUMBERS
DAMD17-98-1-8209

6. AUTHOR(S)
Kristin Roovers, Ph.D.
R. K. Assoian, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

E-Mail: roovers@pharm.med.upenn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Cyclin D1 is frequently overexpressed in breast cancer tumors. Thus, an understanding of cyclin D1 regulation in normal and malignant cells may prove beneficial in the search for therapeutic targets. In fibroblastic cell lines, cyclin D1 expression is regulated by Rho/Rho kinase: i) when Rho/Rho kinase is active, ERK activity is sustained and consequently cyclin D1 is induced in mid-G1 phase, and ii) when Rho/Rho kinase is inactive, cyclin D1 is induce in early G1 phase through a rac-mediated pathway (implicating PI-3 kinase signaling). In this report, I examined the consequences and regulation of rac-mediated cyclin D1 expression in terms of Rho kinase-dependent stress fiber formation. Selective inhibition of Rho kinase allowed for rac-mediated cyclin D1 expression, the downregulation of $p21^{ci\ p1}$ and $p27^{kip1}$ and S phase entry, indicating that cell proliferation can occur under conditions where stress fiber formation is compromised. The suppression of the rac pathway to cyclin D1 expression by Rho kinase did not require stress fiber formation, indicating that there is a Rho kinase effector that has a role in cyclin D1 regulation without affecting actin reorganization. Future work includes the identification of this Rho kinase effector involved in the regulation of rac-mediated cyclin D1 expression.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, cyclin D1, rac, ERK, p21, p27, fibroblastic cell lines, Rho kinase	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

INTRODUCTION

Tumor progression is a multi-step process that ultimately results in uncontrolled proliferation. While the proliferation of non-transformed cells depends on mitogenic stimulation and adhesion to the extracellular matrix (ECM), the proliferation of tumor cells is typically mitogen- and anchorage-independent. Tumor cells have deregulated the signal transduction and cell cycle events controlled by mitogens and adhesion to the ECM. If we understand the normal controls on cell cycle progression, we can begin to define the aberrations that lead to autonomous growth and tumorigenesis.

The G1 phase of the cell cycle is the site of integration of numerous extracellular growth regulatory signals. Progression through G1 phase is controlled by the G1 phase cyclin-dependent kinases (cdks), cdk4 (or cdk6) and cdk2 (1, 2). The activities of these enzymes are determined by the relative levels of their associated cyclins and cyclin-dependent kinase inhibitors. Cyclin D1 is typically induced in mid-G1 phase, where it binds to and activates cdk4 or cdk6. Active cyclin D-cdk4/6 phosphorylates the C-terminal domain of the retinoblastoma protein (pRb), an event that contributes to pRb inactivation by blocking the binding of histone deacetylase (3). The inactivation of pRb, in turn, results in the expression of E2F-regulated genes, including cyclins E and A (4). Cyclin D-cdk4/6 complexes also sequester cip/kip-family cdk inhibitors, thereby contributing to the activation of cyclin E-cdk2 (2). Since the induction of cyclin D1 is often the rate-limiting step in formation of cyclin D-cdk4/6 complexes, the mechanisms that control the induction of cyclin D1 are thought to play a significant role in controlling cell cycle progression through G1 phase.

The extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3-kinase) and the Rho family GTPase, rac signaling pathways have been linked to cyclin D1 expression. The role of ERK in cyclin D1 regulation is well established in several cell types (57). The duration of the ERK signal determines whether cells will express cyclin D1 (8, 9). The induction of cyclin D1 requires activation of ERK in mid-G1 phase (8) and growth factor receptor and integrin signaling synergize to sustain ERK activity into mid-G1 phase (10). PI3-kinase has been reported to contribute to cyclin D1 mRNA induction (11, 12) and cyclin D1 mRNA translation (13). Stimulation of a PI 3-kinase-Akt-GSK-3β pathway results in the stabilization of cyclin D1 protein (14). Others have also observed that activated rac will induce cyclin D1 expression (11, 15, 16) and that this effect is ERK-independent (11, 16). Rac also stimulates the translation of cyclin D1 mRNA, in endothelial cells (17). Since PI 3-kinase can interact with rac either upstream or downstream of the GTPase (18), perhaps PI 3-kinase and rac share a signaling pathway leading to cyclin D1 expression.

We recently reported that the Rho-Rho kinase pathway has a dual function in regulating cyclin D1 gene expression in NIH-3T3 fibroblasts: it allows for sustained ERK activity (defined as activity occurring between 3-9 h after mitogenic stimulation of quiescent cells) and the consequent mid-G1 phase (9 h) induction of cyclin D1 mRNA, while suppressing an alternative rac/cdc42 pathway that leads to an early (1-3 h) G1 phase induction of cyclin D1 mRNA (19). In some reports, inhibition of Rho prevents the expression of cyclin D1 altogether (20) presumably because rac/cdc42 signaling to cyclin D1 mRNA is absent. Thus,

the signaling pathways (ERK vs rac) that a cell can respond to for cyclin D1 expression may have important implications for that cell's prolif eration.

My work in the last 6 months had two main goals: i) to examine the consequences of rac-mediated cyclin D1 induction on G1 phase cell cycle progression, and ii) to identify the pathways that regulate the rac pathway to cyclin D1 expression.

Implications for breast cancer:

Cyclin D1 expression is critical for normal mammary epithelial proliferation. Homozygous deletion of the cyclin D1 gene leads to a reduction in mammary gland development during pregnancy (21), indicating that this tissue lacks a compensatory mechanism for the loss of cyclin D1. Conversely, overexpression of cyclin D1 is linked to the development of breast epithelial tumors. Targeted overexpression of cyclin D1 causes hyperplasia and adenocarcinoma in mammary glands in mice as they reach puberty (22). Also, cyclin D1 is overexpressed in 50-80% of human breast cancers (23). Since only 15% of these can be accounted for by gene amplification, it is interesting to note that several signaling pathways that regulate cyclin D1 expression are defective in tumorigenic breast epithelial. Neu is overexpressed in 20-30% of human breast tumors (24) and cyclin D1 protein levels are increased in response to overexpressed Neu through pathways involving ras, rac, Rho, ERK, JNK and p38 (7). Constitutive activation of NF kappa B nuclear factor (NF-κB) is observed in breast cancer cell lines (25) and has been proposed to control the aberrant cyclin D1 expression (26). And notably, rac-mediated cyclin D1 induction was shown to require NF-κB (27).

BODY OF WORK

Experimental procedures:

Cell culture, transient transfections and inhibitor treatments

MEFs were transiently transfected with plasmids encoding the Rho-binding domain of rhothekin (RBD), dominant negative (CAT-KD) Rho kinase, the p21 binding domain of PAK (PBD), dominant negative LIMK or pCDNA3 (empty vector), serum-starved, seeded in 100-mm culture (~10^6 cells) and stimulated with 10 ml 10% FBS-DMEM as described.[Welsh, 2001 #39] In other experiments, quiescent MEFs were trypsinized, suspended in DMEM-BSA, and pretreated (30 min at 37°C) with the Rho kinase inhibitor, Y-27632 (10 uM; Tocris), U0126 (50 uM; Promega), or DMSO (vehicle) prior to serum-stimulation and reseeding as described above.

NIH-3T3 fibroblasts stably expressing $\alpha5^{human}\beta1^{mouse}$ chimeric integrin (hα5-3T3) have been characterized previously, and the results showed that expression of the chimeric integrin had no effect on the rate G1 phase progression or on the cooperative signaling between RTKs and integrins (10). Quiescent hα5-3T3 cells (1.5-1.8 × 10^6 cells) were suspended in 10 ml serum-free defined medium (10), pretreated (for 30 min at 37°C) with 10 μM ML-7 (Biomol) or DMSO (vehicle) and then stimulated with bFGF (10 ng/ml final concentration; Life Technologies) and immediately plated on 100-mm culture dishes that were coated with fibronectin (Calbiochem; 0.1 mg/10 ml) or anti-$\alpha5\beta1$ (Life Technologies; 1.2 mg/10 ml) as described (10).

Fluorescent microscopy

Quiescent MEFs (2-2.5 x 10^5) were seeded on coverslips in 35-mm dishes with 2 ml 10% FBS-DMEM, fixed and permeabilized (10). Actin was stained with fluorescein-phalloidin (1-1.5 units/ml; 30 min). For vinculin or HA staining, the permeabilized cells were incubated sequentially with anti-vinculin (Sigma; 1:100 dilution for 2 h) or anti-HA (xx; 1:100 dilution for 1 h) and rhodamine-conjugated anti-mouse IgG (Jackson Lab; 1:300 dilution for 1 h). Cell nuclei were stained with Dapi. To analyze S phase entry, the stimulation with 10% FCS was performed in the presence of 3 ug/ml BrdU; permeabilized cells were incubated with DNAase (140 U/ul) and anti-BrdU (BioDesign M20105S; diluted 500-fold; 1 h) and then FITC-conjugated anti-sheep IgG (Jackson Laboratories; diluted 200-fold; 1 h). Images were obtained by epifluoresence microscopy under oil at 40 X magnification, captured using a Hamamatsu digital CCD camera, and analyzed with Openlab Imaging System software.

Extraction and immunoblotting

At specified times following stimulation, cells were washed once with PBS containing 1 mM vanadate, scraped, collected by centrifugation, and quick frozen on dry ice prior to lysis. Frozen cell pellets were lysed as described.[Roovers, 1999 #41] Approximately 60 ug of protein (by Bio-Rad protein assay) were fractionated on reducing SDS-gels (12% acrylamide) and analyzed by western blotting with antibodies specific for cyclin D1 (Santa Cruz, sc-8396), cyclin E (Santa Cruz, sc-481), $p21^{cip1}$ (Santa Cruz, sc-6246), $p27^{kip1}$ (Transduction Lab, K25020), cdk2

(UBI, 06-505), cdk4 (Santa Cruz, sc-260), ERK (Transduction Lab M12320) and phosphoERK (Cell Signaling 9101S).

In vitro cyclin D1-cdk4 and cyclin E-cdk2 kinase assays

Cyclin D1-cdk4 kinase assays using GST-Rb as a substrate were performed as described (19). For cyclin E-cdk2 kinase assays, cell pellets were extracted in 100 µl of freshly prepared lysis buffer (50 mM Tris-HCl pH 8, 250 mM NaCl, 5mM $MgCl_2$, 0.1% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1mM PMSF, 50 mM NaF, 10 mM Na_3VO_4). Equal amounts of cell lysate (250-300 µg in 100 µl lysis buffer) were incubated with 2 µg anti-cyclin E (Santa Cruz, sc-481) for 2 h on ice and then with 50 µl washed protein A-agarose (Invitrogen) for 2 h at 4°C with rocking. Collected immunoprecipitates were washed once with cold lysis buffer and then four times with cold kinase reaction buffer (20 mM HEPES pH 8, 10 mM $MgCl_2$, 0.1 mM DTT, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 mM NaF, 10 mM Na_3VO_4). The washed immunoprecipitates were suspended in 50 µl kinase reaction buffer, containing 1 µg histone H1 (UBI), 10 µM ATP and 20 µCi δ^{32}P-ATP (3000 Ci/mmol). Kinase reactions were stopped with 2 x SDS sample buffer, the samples were fractionated on reducing SDS-gels, and transferred to nitrocellulose membranes. The amount of ^{32}P-histone H1 was visualized by exposure to film. Filters were then immunoblotted with anti-cdk2 to monitor the amount of cdk2 in the cyclin E immunoprecipitates.

Results:

We recently described that in NIH-3T3 fibroblasts, the Rho-Rho kinase pathway is required for sustained ERK activity and thereby helps to restrict the expression of cyclin D1 to mid-G1 phase (10, 19). Moreover, Rho-Rho kinase suppresses an alternative rac/cdc42 pathway that results in the induction of cyclin D1 in early G1 phase (19). These pathways (ERK vs rac) regulating the timing of cyclin D1 expression within G1 phase are parallel and independent and it is Rho kinase activity that determines which pathway is used. The Rho-Rho kinase pathway is best characterized for its positive effect on stress fiber and focal adhesion formation (reviewed in (28). Thus, I decided to examine the regulation and consequences of rac-mediated cyclin D1 expression in the context of stress fiber formation.

Mouse embryo fibroblasts (MEFs) transiently transfected with expression vectors encoding the RBD (a selective Rho inhibitor), dominant negative Rho kinase, or empty vector (control) were rendered quiescent by serum-starvation, and then replated at subconfluence with 10% serum. Alternatively, cells were treated with Y-27632 (a selective pharmacological inhibitor of Rho kinase). In control cells (vector or DMSO) actin was organized into well-defined stress fibers and vinculin staining was punctate characteristic of focal adhesion formation (Fig. 1). As expected, inhibition of either Rho or Rho kinase blocked the formation of actin stress fibers and punctate vinculin staining (Fig. 1).

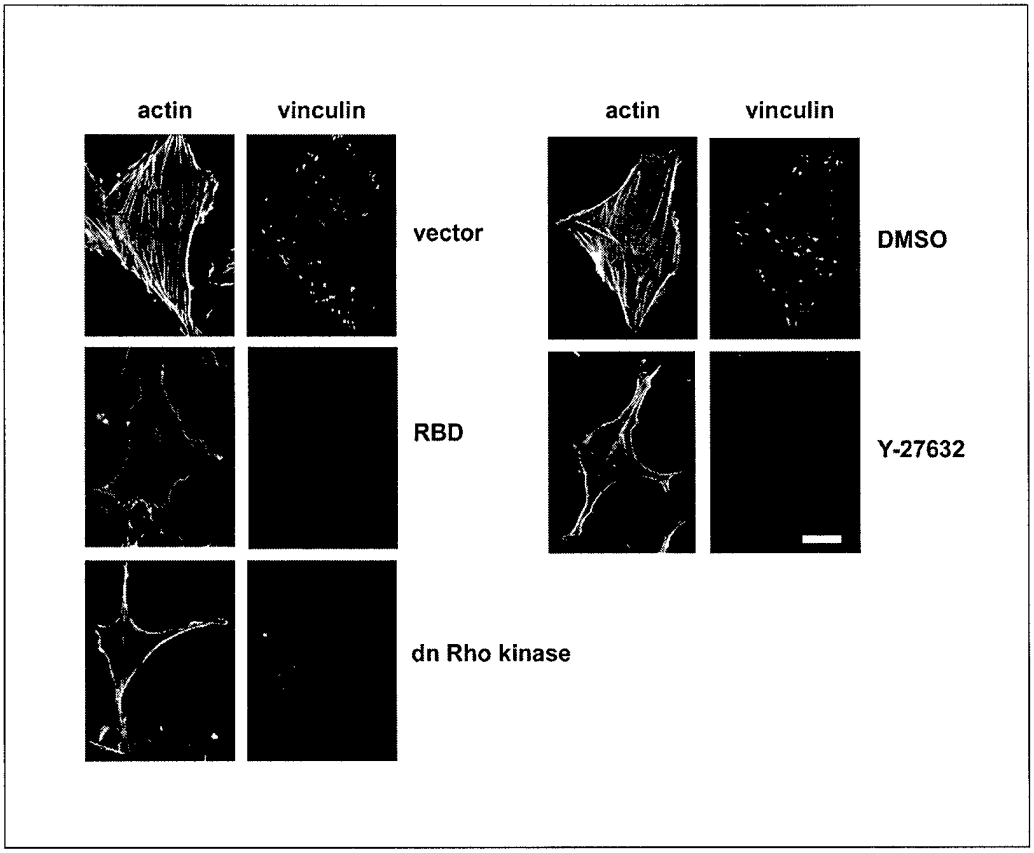

Figure 1. *Inhibition of Rho/Rho kinase disrupts stress fiber formation. MEFs were transiently transfected [with empty vector, the RBD expression vector, or a dominant negative (dn) Rho kinase expression vector] and then serum-starved, or serum-starved and then pretreated with DMSO (vehicle) or 10 μM Y-27632. The cells were plated at subconfluence in dishes containing coverslips, and stimulated with 10% FBS. Coverslips were collected at 18 h, fixed, permeabilized and analyzed for f-actin and vinculin by fluorescence microscopy (bar = 5 μm).*

Consistent with our results in NIH-3T3 fibroblasts, I found that inhibition of either Rho or Rho kinase in MEFs blocked sustained ERK activity (3-9 h) and allowed for the induction of cyclin D1 in early G1 phase (Fig. 2A and B; 3 h). Inhibition of either Rho or Rho kinase also led to the formation of catalytically active cyclin D1-cdk4 complexes in early G1 phase (Fig. 2B; 3 h). The mid-G1 phase induction of cyclin D1 was blocked by inhibition of MEK/ERK signaling with U0126 but resistant to inhibition of rac/cdc42 signaling by the p21-binding domain of PAK (PBD) (Fig. 2C, compare control, U0126, and PBD; 9h -Y-27632). Conversely, the early G1 phase expression of cyclin D1 seen in Rho kinase-inhibited cells was unaffected by U0126 and blocked by expression of the PBD (Fig. 2C, compare control, U0126, and PBD; 3 and 9 h +Y-27632). These results show that ERK-dependent cyclin D1 expression is dependent on Rho kinase-mediated stress fiber formation while rac/cdc42-dependent cyclin D1 expression proceeds normally even after disruption of Rho kinase-dependent stress fibers.

In general, cell proliferation is thought to dependent upon organization of the actin cytoskeleton. Studies with inhibitors of actin polymerization and targeted disruption of isometric tension implicate actin-dependent stress fiber formation in cycle progression through

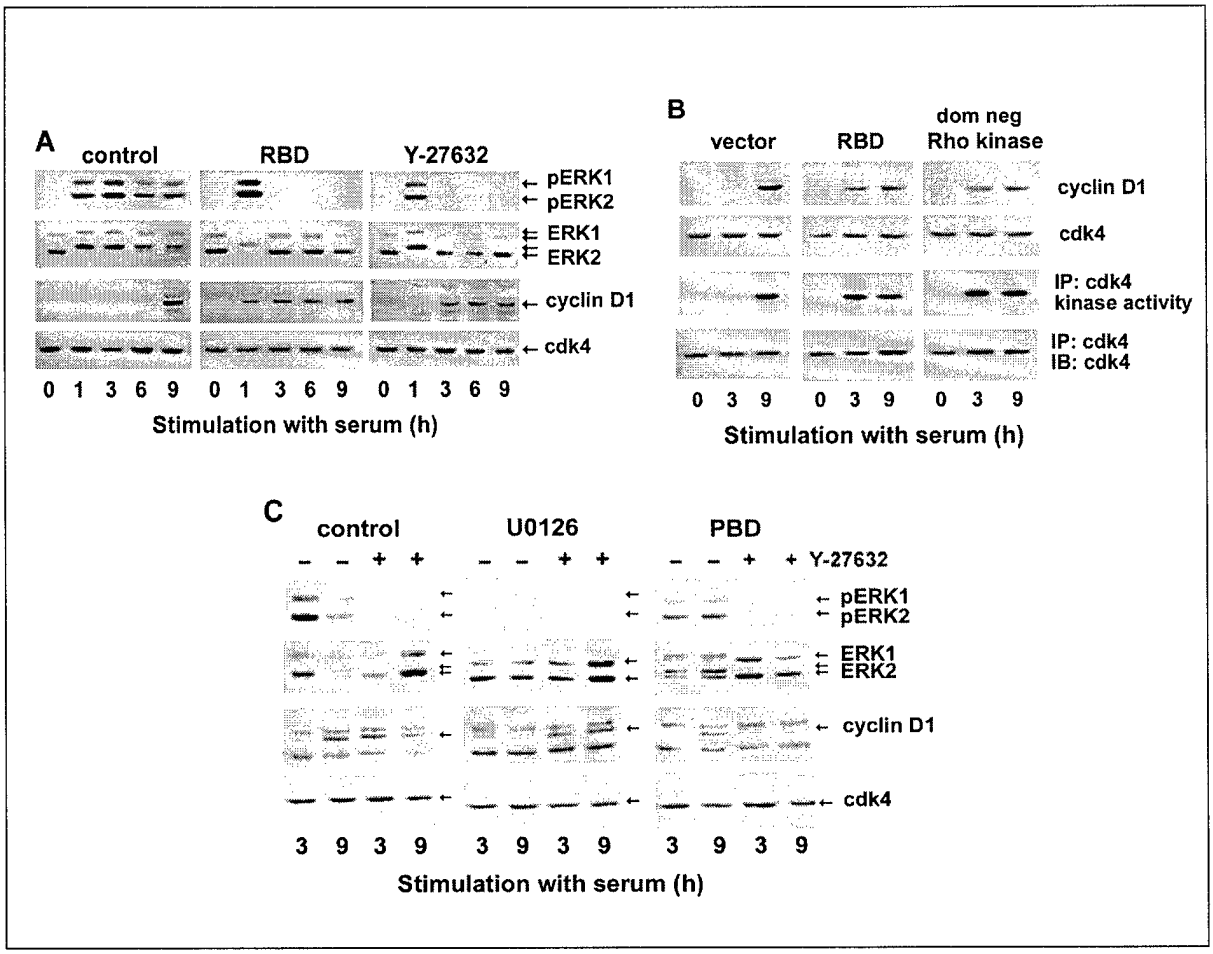

Figure 2. Rac/cdc42-mediated cyclin D1 expression occurs in the absence of stress fibers. In A, serum-starved MEFs transiently transfected with the RBD expression vector or pretreated with Y-27632 were plated at subconfluence and stimulated with 10% FBS. Collected cells were analyzed for the activation of ERK by immunoblotting with an antibody specific for dually phosphorylated ERK1 and ERK2 or by gel-shift with anti-ERK (the upper arrows in each set indicate the activated ERK) and for the expression of cyclin D1 and cdk4 (loading control). In B, MEFs transiently transfected with empty vector, the RBD expression vector or the dominant negative (dom neg) Rho kinase expression vector were stimulated with 10% FBS. Collected cells were lysed and analyzed for the expression of cyclin D1 and cdk4 or incubated with anti-cdk4 and immunoprecipitates were used to assess in vitro cdk4 kinase activity by phosphorylation of GST-Rb protein. The amount of immunoprecipitated (IP) cdk4 was assessed by immunoblotting (IB) using filters from the kinase assay. In C, MEFs transiently transfected with empty vector (control) or the PBD expression vector were serum-starved and preincubated with 50 uM U0126 (Promega), 10 uM Y-27632 or both U0126 and Y-27632. The cells were then reseeded at subconfluence, stimulated with 10% FBS and analyzed as described for panel A.

G1 phase, particularly cyclin D1 expression and the downregulation of $p21^{cip1}$ and/or $p27^{kip1}$ (6, 29). Therefore, I asked what consequence rac-mediated cyclin D1 expression (which occurs in the absence of stress fibers) has on G1 phase cell cycle progression.

Consistent with studies by others (30), inhibition of Rho signaling prevented the downregulation of both $p21^{cip1}$ and $p27^{kip1}$ that normally occur in mid-late G1 phase (Fig. 3A). The role of Rho kinase in regulating $p21^{cip1}$ and $p27^{kip1}$ levels has yet to be resolved (31, 32), but in agreement with Sahai et al. (32), we found that inhibition of Rho kinase did not prevent the downregulation of $p21^{cip1}$ and $p27^{kip1}$ (Fig. 3A; 9 and 15 h). Furthermore, cyclin E-cdk2 activity was blocked when Rho was inhibited whereas cyclin E-cdk2 complexes were activated in both control and Rho kinase-inhibited cells (Fig. 3B). Thus, Rho

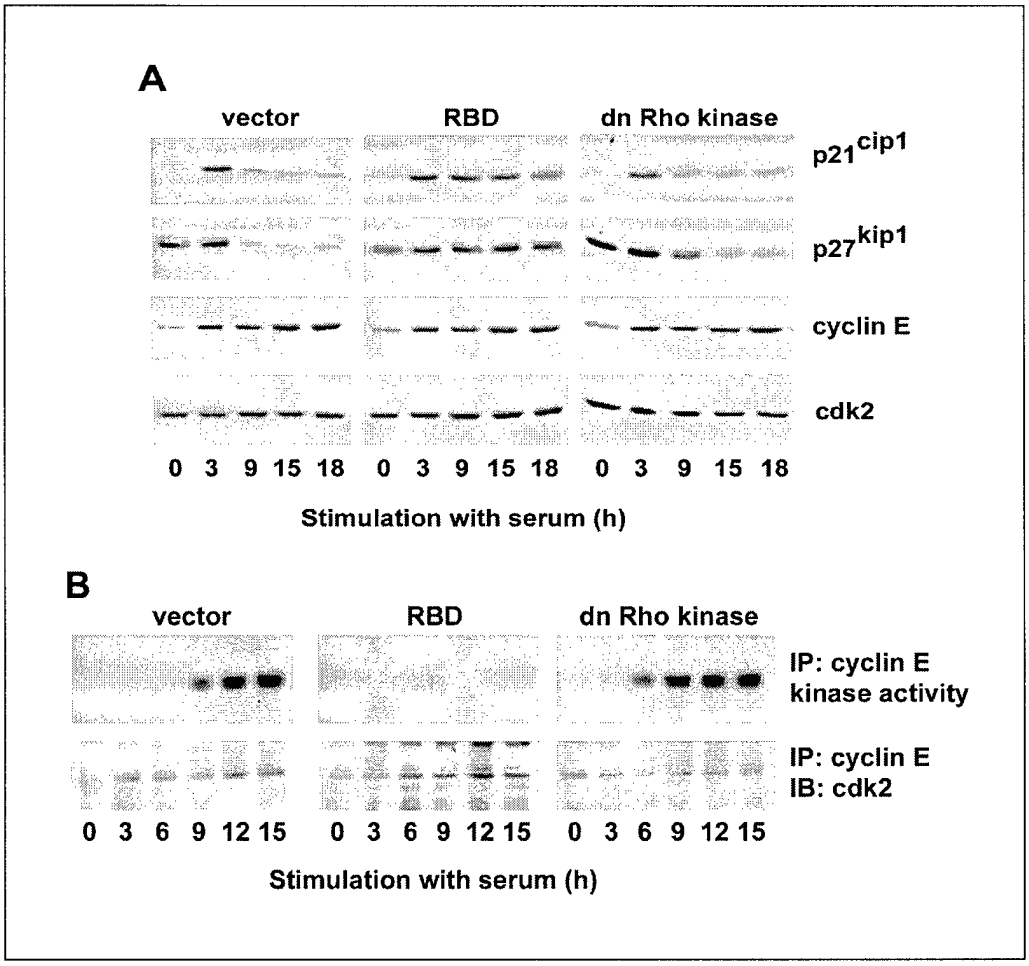

Figure 3. *Rho kinase inhibition allows for $p21^{cip1}$ and $p27^{kip1}$ downregulation and cyclin E-cdk2 activity. Serum-starved MEFs transiently transfected with empty vector, the RBD expression vector, or the dominant negative (dn) Rho kinase expression vector were plated at subconfluence and stimulated with 10% FBS. In A, collected cells were lysed and were analyzed by immunoblotting using antibodies specific for $p21^{cip1}$, $p27^{kip1}$, cyclin E or cdk2 (loading control). In B, cell lysates were incubated with anti-cyclin E, and the collected immunoprecipitates (IP) were used to assess in vitro cyclin E-cdk2 kinase activity by phosphorylation of histone H1. The levels of cyclin E-associated cdk2 was determined by immunoblotting (IB) using filters from the kinase assay.*

kinase-dependent stress fiber formation is not required for the downregulation of $p21^{cip1}$ and $p27^{kip1}$ or for the activation of cyclin E-cdk2 in MEFs. In fact, Rho kinase inhibition even led to

the activation of cyclin E-cdk2 earlier in G1 phase when compared to control cells (Fig. 3B; 6 h). Since Rho kinase inhibition does not alter the levels of cyclin E, cdk2, or its inhibitors (Fig. 3A), the premature activation of cyclin E-cdk2 is the likely consequence of $p21^{cip1}$ and $p27^{kip1}$ sequestration by the early G1 phase cyclin D1-cdk4 complexes (2). Together, Figs. 2 and 3 indicate that the only function of stress fibers in MEFs is to sustain ERK activity and thereby permit the mid-G1 phase induction of cyclin D1.

Quiescent MEFs transiently transfected with RBD or dominant negative Rho kinase were then serum-stimulated at subconflence to assess the effects of Rho and Rho kinase inhibition on cell cycle progression into S phase. Rho inhibition blocked S phase entry (Fig. 4A, compare vector and RBD), but inhibition of Rho kinase accelerated S phase entry by ~4 h (Fig. 4, compare vector and dn Rho kinase). A similar ~4 h acceleration of S phase entry was observed when Rho kinase was inhibited with Y-27632 (Fig. 4). Both flow cytometry and immunoblotting for the induction of cyclin A (a marker of S phase entry) also demonstrated a similar ~ 4 h shortening of G1 phase upon inhibition of Rho kinase (not shown). Thus, when Rho kinase is inhibited, the premature activation of cyclin D1-cdk4 and cyclin E-cdk2 is associated with a shortening of G1 phase. This finding -- that the duration of G1 phase is actually decreased upon inhibition of Rho kinase -- strongly argues that a small number of residual stress fibers does not account for the absence of G1 phase arrest in Rho kinase-inhibited MEFs. The accompanying change in cyclin D1 regulation, from sustained ERK to rac/cdc42, also supports this conclusion.

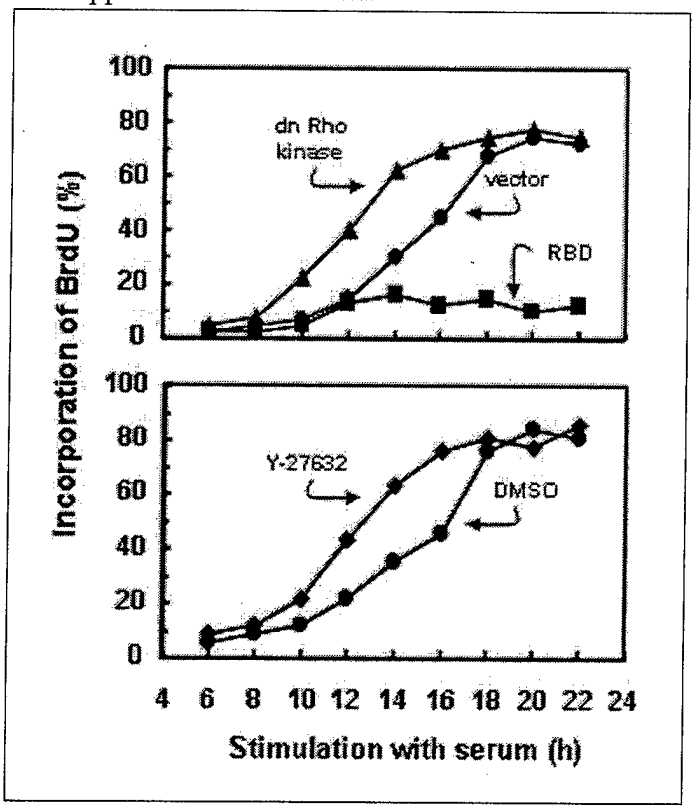

Figure 4. *Inhibition of Rho kinase supports G1 phase cell cycle progression and entry into S phase. MEFs were transiently transfected [with empty vector, the RBD expression vector, or a dominant negative (dn) Rho kinase expression vector] and then serum-starved, or serum-starved and then pretreated with DMSO (vehicle) or 10 μM Y-27632. The cells were plated at subconfluence in dishes containing coverslips, and stimulated with 10% FBS. Coverslips were collected and fixed at the indicated times for an analysis of S phase entry by BrdU incorporation.*

The G1 arrest seen upon inactivation of Rho, even when cyclin D1 is expressed, is consistent with the idea that actin stress fiber formation is required for cell cycle progression (19). However, inhibition of Rho has effects that go well beyond the disruption of stress fibers (33). The specific inhibition of Rho kinase allows for rac-mediated

cyclin D1 induction, the downregulation $p21^{cip1}$ and $p27^{kip1}$, and S phase entry. Thus, my data show that Rho kinase-dependent stress fibers are not intrinsically required for G1 phase cell cycle progression.

Since the loss of stress fibers (i.e. Rho or Rho kinase inhibition) is associated with rac-mediated cyclin D1 induction, I next asked whether the suppression of early cyclin D1 expression by rac was specifically attributable to inhibition of stress fiber formation. Stress fiber formation is largely regulated by myosin light chain (MLC) phosphorylation which is mediated by MLC kinase (MLCK) and promotes both myosin filament assembly and actin-activated myosin ATPase activity (34). I inhibited stress fiber formation by treating hα5-3T3 fibroblasts with ML-7 (a selective inhibitor of MLCK; Fig. 5A). Cyclin D1 expression was completely blocked by ML-7 (Fig. 5B) which, although consistent with the block in sustained ERK activity, indicates that disruption of stress fibers per se does not relieve the suppression on the rac-mediated pathway to cyclin D1.

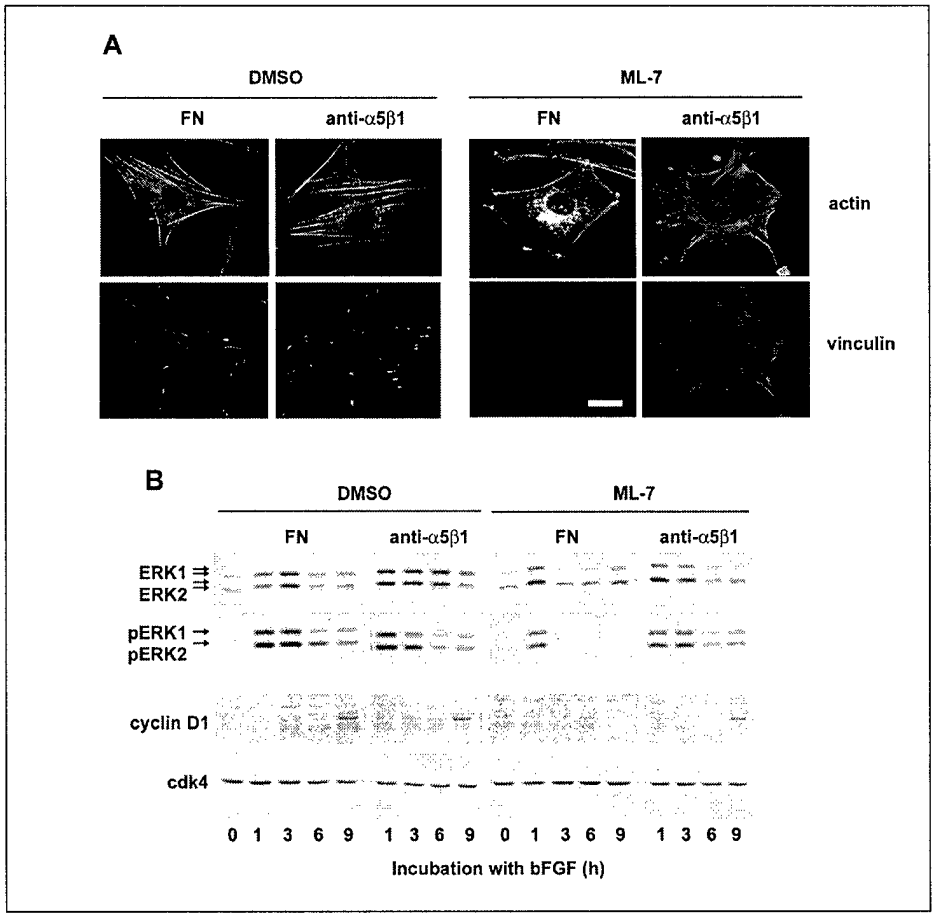

Figure 5. *Stress fibers are required to cluster integrins for sustained ERK activity but they are not involved in suppressing the rac pathway to cyclin D1 expression. Quiescent hα5-3T3 fibroblasts were pretreated with DMSO or ML-7, seeded on fibronectin (FN) or anti-α5β1-coated dishes containing coverslips and stimulated with bFGF. In A, cells were fixed after 6 h and stained for f-actin and vinculin. Scale bar = 5 μm. In B, cells were collected at the indicated times, lysed, and analyzed for activation of ERK by gel-shift of ERK1 and 2 (arrows) and by direct detection of dually phosphorylated ERKs (pERK). Cell lysates were also analyzed for cyclin D1 expression and cdk4 (loading control) by immunoblotting.*

Interestingly, when ML-7-treated cells were plated on anti-α5β1, stress fiber formation remained efficiently inhibited but punctate vinculin staining was restored (Fig. 5A) and rescued

sustained ERK activity and mid-G1 phase cyclin D1 expression. Since vinculin interacts with β1 integrin tails (35), these data strongly argue that plating cells on anti-α5β1 allowed for integrin clustering even in the absence of stress fibers. The effect of anti-α5β1 on ERK signal duration was not blocked by cycloheximide, indicating that it was not a consequence of secreted matrix proteins, and required bFGF, indicating that the preparation of anti-α5β1 was not mitogenic in itself (10); our unpublished data). Thus, integrin clustering is required to sustain ERK activity.

My results indicate that, while stress fibers regulate sustained ERK activity by acting upstream of integrins to maintain their clustering and signaling, stress fibers are not involved in the suppression of rac-mediated pathway to cyclin D1 induction. Therefore, there is a Rho kinase effector that functions to suppress the rac pathway to cyclin D1 expression independently of stress fiber formation. I am currently working to identify this Rho kinase effector and further elucidate the mechanism responsible for the regulation of rac-mediated cyclin D1 induction.

Key Research Accomplishments:

- Rho kinase-dependent stress fibers are required for ERK-dependent induction of mid-G1 phase cyclin D1 but not for rac/cdc42-dependent induction of early G1 phase cyclin D1.
- Selective disruption of Rho kinase-dependent stress fibers does not inhibit G1 cell cycle progression.
- The effect of stress fibers on integrin clustering explains how the Rho-Rho kinase pathway regulates sustained ERK activity.
- Stress fibers do not suppress the rac-mediated pathway to cyclin D1 expression, indicating that a Rho kinase effector has a role in cyclin D1 regulation, independent of stress fiber formation.

Reportable outcomes:

a) manuscripts: one, recently submitted for review
b) publications: none
c) abstracts: one
d) patents and licenses applied for: none
e) degrees obtained: none
f) development of cell lines: none
g) funding applied for: none
h) employment opportunities applied for: none

Conclusions:

My work demonstrated that Rho kinase inhibition allows for rac-mediated cyclin D1 expression and, despite the loss of stress fibers, supports G1 phase cell cycle progression. While stress fibers are required for sustained ERK-dependent cyclin D1 expression, they are not involved in the suppression of the rac pathway to cyclin D1 induction by Rho kinase. The use of different signal transduction mechanisms regulating cyclin D1 (rac/cdc42 vs. sustained ERK) can confer distinct proliferative properties to cells. For example, selective activation of rac/cdc42 signaling might allow for cell proliferation under conditions where adhesion-dependent formation of stress fibers is compromised, which may be an important step in tumorigenesis. An understanding of the complex signal transduction pathways involved in the regulation of cyclin D1 expression in normal cells will allow us to define the aberrant pathways in tumor cells. Presently, my work is focused on elucidating the mechanism by which Rho kinase suppresses the rac-mediated induction of cyclin D1. In the future, the lab will examine the signaling pathways involved in the regulation of cyclin D1 induction in normal and tumorigenic breast cell lines, using the model established in fibroblasts as a framework. This research may provide a target signaling molecule that when interfered with would ablate the overexpressed cyclin D1 that is so prevalent in malignant breast epithelial cells.

References

1. C. J. Sherr, *Cell* 79, 551-5 (1994); R. A. Weinberg, *Cell* 81, 323-30. (1995).

2. C. J. Sherr, J. M. Roberts, *Genes Dev* 13, 1501-12 (1999).

3. J. W. Harbour, R. X. Luo, A. Dei Santi, A. A. Postigo, D. C. Dean, *Cell* 98, 859-69. (1999).

4. J. DeGregori, T. Kowalik, J. R. Nevins, *Mol Cell Biol* 15, 4215-24. (1995).

5. C. Albanese *et al.*, *J Biol Chem* 270, 23589-97 (1995); M. Cheng, V. Sexl, C. J. Sherr, M. F. Roussel, *Proc Natl Acad Sci U S A* 95, 1091-6 (1998); J. N. Lavoie, G. L'Allemain, A. Brunet, R. Muller, J. Pouyssegur, *J Biol Chem* 271, 20608-16 (1996); D. Woods *et al.*, *Mol Cell Biol* 17, 5598-611. (1997).

6. S. Huang, D. E. Ingber, *Exp Cell Res* 275, 255-64. (2002).

7. R. J. Lee *et al.*, *Mol Cell Biol* 20, 672-83. (2000).

8. J. D. Weber, D. M. Raben, P. J. Phillips, J. J. Baldassare, *Biochem J* 326, 61-8. (1997).

9. K. Balmanno, S. J. Cook, *Oncogene* 18, 3085-97. (1999).
10. K. Roovers, G. Davey, X. Zhu, M. E. Bottazzi, R. K. Assoian, *Mol Biol Cell* 10, 3197-204. (1999).

11. H. Gille, J. Downward, *J Biol Chem* 274, 22033-40. (1999).

12. N. Takuwa, Y. Fukui, Y. Takuwa, *Mol Cell Biol* 19, 1346-58. (1999).

13. R. C. Muise-Helmericks *et al.*, *J Biol Chem* 273, 29864-72. (1998).

14. J. A. Diehl, M. Cheng, M. F. Roussel, C. J. Sherr, *Genes Dev* 12, 3499-511. (1998).

15. J. K. Westwick *et al.*, *Mol Cell Biol* 17, 1324-35. (1997); O. Gjoerup, J. Lukas, J. Bartek, B. M. Willumsen, *J Biol Chem* 273, 18812-8. (1998).

16. K. Page *et al.*, *J Biol Chem* 274, 22065-71. (1999).

17. A. Mettouchi *et al.*, *Mol Cell* 8, 115-27. (2001).

18. G. Scita *et al.*, *Embo J* 19, 2393-8. (2000); X. D. Ren, M. A. Schwartz, *Curr Opin Genet Dev* 8, 63-7. (1998).

19. C. F. Welsh *et al.*, *Nat Cell Biol* 3, 950-7. (2001).

20. E. H. Danen, P. Sonneveld, A. Sonnenberg, K. M. Yamada, *J Cell Biol* 151, 1413-22. (2000); L. K. Hansen, D. J. Mooney, J. P. Vacanti, D. E. Ingber, *Mol Biol Cell* 5, 967-75. (1994).

21. R. L. Sutherland, E. A. Musgrove, *Breast Cancer Res* 4, 14-7 (2002).

22. T. C. Wang *et al.*, *Nature* 369, 669-71. (1994).

23. P. L. Fernandez, P. Jares, M. J. Rey, E. Campo, A. Cardesa, *Mol Pathol* 51, 305-9. (1998); D. M. Barnes, *J Pathol* 181, 267-9. (1997); J. Bartkova *et al.*, *Int J Cancer* 57, 353-61. (1994); M. F. Buckley *et al.*, *Oncogene* 8, 2127-33. (1993).

24. H. Lacroix, J. D. Iglehart, M. A. Skinner, M. H. Kraus, *Oncogene* 4, 145-51. (1989); D. J. Slamon *et al.*, *Science* 235, 177-82. (1987).

25. H. Nakshatri, P. Bhat-Nakshatri, D. A. Martin, R. J. Goulet, Jr., G. W. Sledge, Jr., *Mol Cell Biol* 17, 3629-39. (1997).

26. Y. Cao *et al.*, *Cell* 107, 763-75. (2001).

27. D. Joyce *et al.*, *J Biol Chem* 274, 25245-9. (1999).
28. S. T. Barry, H. M. Flinn, M. J. Humphries, D. R. Critchley, A. J. Ridley, *Cell Adhes Commun* 4, 387-98. (1997); E. A. Clark, W. G. King, J. S. Brugge, M. Symons, R. O. Hynes, *J Cell Biol* 142, 573-86. (1998); A. Hall, *Science* 279, 509-14. (1998).

29. R. M. Bohmer, E. Scharf, R. K. Assoian, *Mol Biol Cell* 7, 101-111. (1996); M. E. Bottazzi *et al.*, *Mol Cell Biol* 21, 7607-16. (2001); J. Fringer, F. Grinnell, *J Biol Chem* 276, 31047-52. (2001); S. Huang, C. S. Chen, D. E. Ingber, *Mol Biol Cell* 9, 3179-93. (1998); H. Rosenfeldt, F. Grinnell, *J Biol Chem* 275, 3088-92. (2000).

30. J. Adnane, F. A. Bizouarn, Y. Qian, A. D. Hamilton, S. M. Sebti, *Mol Cell Biol* 18, 6962-70. (1998); K. L. Auer *et al.*, *Biochem J* 336, 551-60. (1998); W. Hu, C. J. Bellone, J. J. Baldassare, *J Biol Chem* 274, 3396-401. (1999); U. Laufs, D. Marra, K. Node, J. K. Liao, *J Biol Chem* 274, 21926-31. (1999); M. F. Olson, H. F. Paterson, C. J. Marshall, *Nature* 394, 295-9. (1998); J. D. Weber, W. Hu, S. C. Jefcoat, Jr., D. M. Raben, J. J. Baldassare, *J Biol Chem* 272, 32966-71. (1997).

31. H. Iwamoto *et al.*, *J Hepatol* 32, 762-70. (2000).

32. E. Sahai, T. Ishizaki, S. Narumiya, R. Treisman, *Curr Biol* 9, 136-45. (1999); E. Sahai, M. F. Olson, C. J. Marshall, *Embo J* 20, 755-66. (2001).

33. A. L. Bishop, A. Hall, *Biochem J* 348 Pt 2, 241-55. (2000); L. Van Aelst, C. D'Souza-Schorey, *Genes Dev* 11, 2295-322. (1997).

34. K. Burridge, M. Chrzanowska-Wodnicka, *Annu Rev Cell Dev Biol* 12, 463-518 (1996).

35. J. M. Lewis, M. A. Schwartz, *Mol Biol Cell* 6, 151-60. (1995); S. Miyamoto, S. K. Akiyama, K. M. Yamada, *Science* 267, 883-5. (1995); G. Plopper, D. E. Ingber, *Biochem Biophys Res Commun* 193, 571-8. (1993).

APPENDIX

Abstract presented at Gordon Research Conference 2002 – Signaling by Adhesion Receptors

G1 phase cell cycle progression in the absence of Rho kinase-dependent stress fibers

Kristin Roovers and Richard K. Assoian

We previously demonstrated that Rho/Rho kinase controls the timing of cyclin D1 expression within G1 phase of fibroblasts. Rho kinase is involved in sustaining the ERK activity required for mid-G1 cyclin D1 expression and it also suppresses a rac/cdc42-mediated pathway leading to early G1 phase cyclin D1 expression. We now report that although inhibition of either Rho or Rho kinase allowed rac/cdc42-induced cyclin D1 expression and cdk4 activity in early G1 phase, cells with Rho inhibited arrested in G1 phase (due to an inhibition of cdk2 activity), whereas cells with Rho kinase inhibited effectively activated cdk2 and progressed into S phase. Thus, selective targeting of Rho kinase demonstrates that stress fibers are not intrinsically required for G1 phase cell cycle progression. The consequence of stress fiber formation and disruption depends on the relative importance of ERK vs. rac/cdc42 signaling in the induction of cyclin D1. The apparent redundancy in the signal transduction systems regulating cyclin D1 actually allows for proliferation under distinct growth conditions.